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Original Article

Immunohistochemical expression of RUNX3 and p21$^{WAF1/CIP1}$ and its correlation with clinicopathologic parameters in breast carcinoma of Egyptian women

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Abstract: Background: RUNX3 is a tumor suppressor involved in many cancers. RUNX3 regulation is complex; it can both repress and activate p21$^{WAF1/CIP1}$. The cyclin-dependent kinase inhibitor p21^{waf1} is a key regulator of progression from the G1 to the S-phase of cell cycle. Previous studies on the prognostic value of RUNX3 and p21^{waf1} in breast carcinoma revealed conflicting results. To date, no immunohistochemical studies have been performed to assess the possible association between them in breast carcinoma. The aim of this study is to evaluate the immunohistochemical expression of RUNX3 and p21^{waf1} and investigate its association with clinicopathological prognostic parameters and to assess the possible relationship between them. Methods: A retrospective immunohistochemical study was conducted on 78 invasive ductal breast carcinoma cases using RUNX3 and p21^{waf1}. Statistical analysis was performed. Results and Conclusion: The current study reports for the first time the relationship between RUNX3 and p21^{waf1} immunohistochemical expression in breast cancer; there was a highly statistically significant relation between RUNX3 and p21^{waf1} expressions (P=0.001), and a moderate significant agreement (kappa =0.467) between them. The two markers are statistically related to established prognostic clinicopathologic parameters suggesting a role in prognosis; RUNX3 showed high statistically significant relations with grade and lymph nodal status (P=0.001 for each), and statistically significant relations with tumor size and molecular subtypes (P=0.02, and P=0.04 respectively). p21^{waf1} showed highly statistically significant relations with grade, lymph nodal status, ER expression, PR expression, and molecular subtypes (P=0.001 for each), and a statistically significant relation with tumor size (P=0.045). The two markers also represent potential therapeutic targets for patients with breast cancer.

Keywords: Breast carcinoma, RUNX3, p21^{WAF1}, immunohistochemistry

Introduction

Breast cancer is the most common malignancy that affects women, with a prevalence of 23% of all cancers in women worldwide. It is the second most common cause of death in women after lung cancer [1, 2]. In Egypt, carcinoma of the breast is the most prevalent cancer among Egyptian women and constitutes 17.5% of all malignant tumors presented to National Cancer Institute, Cairo University, in the years 2003-2004 [3]. A variety of clinical and pathological factors are routinely used to categorize patients with breast cancer in order to assess prognosis and determine the appropriate therapy. These include patient age, axillary lymph node status, tumor size and grade, hormone receptor status, and Her2 status. Although these risk categories are useful for assessing prognosis and risk in groups of patients with breast cancer, their role in determining prognosis and evaluating risk in an individual patient is more limited. Therefore, better methods are required to help assess prognosis and determine the most appropriate treatment for patients on an individual basis. Various molecular techniques have been increasingly used to help refine breast cancer classification and to assess prognosis and response to therapy [4].

The Runt family of three transcription factors (RUNX1, 2 and 3) is known to play essential roles in haematopoiesis, osteogenesis and neurogenesis [5]. Besides being key developmental regulators, RUNX genes are also important in cancer, acting both as oncogenes or

tumor suppressors in different systems [6]. Among the three RUNX family members, RUNX3 was first suggested to be a tumor suppressor due to causal relationship between the loss of RUNX3 and the progression of gastric cancer [7]. Since the discovery of the potential role of RUNX3 in the initiation and progression of gastric cancer, RUNX3 has been found to be involved in the development of various cancers, including colorectal cancer, liver cancer, lung cancer, and breast cancer [8, 9].

A striking observation that highlights the complexity of RUNX3 regulation and provides us with another potential insight into its behavior in cancer is that RUNX3 can both repress and activate p21$^{WAF1/CIP1}$. p21$^{WAF1/CIP1}$ was first identified as a low-molecular-weight protein in cyclin-dependent kinase (CDK) and cyclin immunocomplexes involved in connecting various cellular pathways to cell cycle control. Historically, p21$^{WAF1/CIP1}$ has been accepted as a CDK inhibitor that ultimately leads to E2F transcription factor sequestration and the arrest of cellular proliferation at the G1 restriction point. Nevertheless, p21$^{WAF1/CIP1}$ expression has been shown to be positively associated with survival in gastric, anal, prostate, and breast cancers [10, 11].

To the best of my knowledge, no immunohistochemical studies have been performed to assess the possible association between RUNX3 and p21$^{WAF1/CIP1}$ in breast carcinoma. This study is designed to correlate RUNX3 and p21$^{WAF1/CIP1}$ with clinicopathological prognostic parameters in breast carcinoma and assess the relationship between these two proteins.

Materials and methods

Tissue and patient data

The current study was conducted on 78 cases of invasive ductal breast carcinoma which is the most common subtype of breast carcinomas. Cases were obtained from the Archives of the Pathology Lab. of Ain-Shams University Specialized Hospital. Such cases were diagnosed during the period from January 2012 to January 2014. They were obtained by modified radical mastectomy. The surgical and histopathological reports were reviewed to determine age of patients, tumor size (greatest dimension), estrogen-receptor (ER), progesterone-receptor (PR) and HER2 status, as well as lymph nodal involvement. For each patient, clinical stage at presentation was classified according to the 2003 American Joint Committee on Cancer Staging System [12]. Hematoxylin and Eosin stained slides were examined to re-evaluate and verify the histopathological diagnosis and grade (according to the modified Bloom and Richardson method [13]. Only cases with information for all the covariates were selected in the analysis.

Ethics statement

All patients who participated in this study signed a written, informed consent before surgery. The study was approved by the Research Ethical Committee at Faculty of Medicine, Ain Shams University.

Immunohistochemical staining

Four micrometer sections of formalin-fixed and paraffin-embedded samples of 78 breast carcinoma cases were prepared. Immunohistochemical staining was performed using primary antibodies; mouse monoclonal anti-RUNX3 antibody (Clone: (R3-5G4) sc-101553; Santa Cruz Biotechnologies, Santa Cruz, Calif) and mouse monoclonal anti p21^{waf1} antibody (Clone: 4D10; Novocastra Laboratories, Newcastle). Avidin-Biotin immunoperoxidase complex technique was used according to Hsu et al. [14] by applying the super sensitive detection kit (Biogenex, CA, USA). The prepared tissue sections were fixed on poly-L-lysine coated slides overnight at 37°C. They were deparaffinized and rehydrated through graded alcohol series. Then the sections were heated in a microwave oven in 10 mM citrate buffer (pH 6.0) for 20 min. After the blocking of endogenous peroxidase and incubation in Protein Block Serum-Free Solution (Dako Cytomation) for 20 min, the sections were incubated overnight at 4°C with primary antibodies. Biotinylated anti- mouse immunoglobulin and streptavidin conjugated to horseradish peroxidase were then added. Finally, 3,3'-diaminobenzidine as the substrate or chromogen was used to form an insoluble brown product. Finally, the sections were counterstained with hematoxylin and mounted. Sections of human stomach tissue and colorectal carcinoma were used as positive control for

Table 1. Clinicopathological characteristics of patients with breast carcinoma (n=78)

		N	%
Age	≤50 years	23	29.5%
	>50 years	55	70.5%
Grade	I	14	17.9%
	II	32	41.0%
	III	32	41.0%
Lymph node status	Negative	42	53.8%
	Positive	36	46.2%
Size	≤20 mm	45	57.7%
	21-50 mm	30	38.5%
	>50 mm	3	3.8%
ER	Negative	36	46.2%
	Positive	42	53.8%
PR	Negative	36	46.2%
	Positive	42	53.8%
HER2	Negative	60	76.9%
	Positive	18	23.1%
Molecular type	Triple negative	23	29.5%
	Her2 + only	13	16.7%
	Lum	37	47.4%
	Luminal Her2	5	6.4%
RUNX3	Negative	14	17.9%
	Positive	64	82.1%
RUNX3 degree of expression	Low	40	62.5%
	High	24	37.5%
P21^{waf1}	Negative	20	25.6%
	Positive	58	74.4%
P21^{waf1} degree of expression	Low	38	65.5%
	High	20	34.5%

Triple negative phenotypic cases [ER-, PR-, and HER2-] referred to as TNP, HER2+/ER-PR-subtype, Luminal' (LUM) subtype [ER+ and/or PR+ plus HER2-], Luminal/HER2+ (LUM/HER2+) subtype [ER+ and/or PR+ plus HER2+].

RUNX3 and p21^{waf1} respectively. Negative control sections were incubated with normal mouse serum instead of the primary antibody.

Interpretation of immunohistochemical staining

Immunohistochemical analysis of RUNX3 and p21^{waf1} was blindly performed by the author without any prior knowledge of the clinicopathological data. For both markers, all tumor cells with detectable nuclear staining were considered to be positive. RUNX3 protein expression as well as p21^{waf1} protein expression was assessed by semi- quantitative scoring of the intensity of staining according to the percentage of positive cells in at least five areas at a magnification of 400×. All tumor cells with detectable nuclear staining was considered to be positive. For statistical analysis, the samples were divided according to percent distribution of stained tumor cell nuclei into low and high expression groups using the median value as a cutoff point. The cutoff values were as follows: Both RUNX3 and p21^{waf1}, low <10 and high ≥10 [15-18].

Using immunohistochemistry for ER, PR, and HER2 as a surrogate for expression profiling, the studied tumors were classified according to Cheang et al. [19] as follows: (a) Triple negative phenotypic cases [ER-, PR-, and HER2-] referred to as TNP; (b) HER2+/ER-PR- subtype; (c) Luminal' (LUM) subtype [ER+ and/or PR+ plus HER2-]; (d) Luminal/HER2+ (LUM/HER2+) subtype [ER+ and/or PR+ plus HER2+]. Luminal/HER2+ is not synonymous with the luminal B expression profile subtype because only 30% to 50% of luminal B tumors express HER2. Luminal' includes all cases that expression profiling defines as luminal A, as well as those remaining luminal B tumors that do not express HER2 [19].

Statistical analysis

Continuous variables are expressed as mean and Standard Deviation. Categorical variables are expressed as frequencies and percent. Student T Test was used to assess the statistical significance of the difference between two study group means. Chi-Square and fisher exact were used to examine the relationship between two qualitative variables. Kappa statistics was used to compute the measure of agreement between RUNX3 and p21^{waf1}; Kappa's values <0 indicated no agreement while 0-0.20 indicated slight agreement, 0.21-0.40: fair, 0.41-0.60: moderate, 0.61-0.80: strong and 0.81-1 revealed almost perfect agreement. P<0.05 was considered the cut-off value of significance. All statistical procedures were carried out using SPSS version 15 for Windows (SPSS Inc, Chicago, IL, USA).

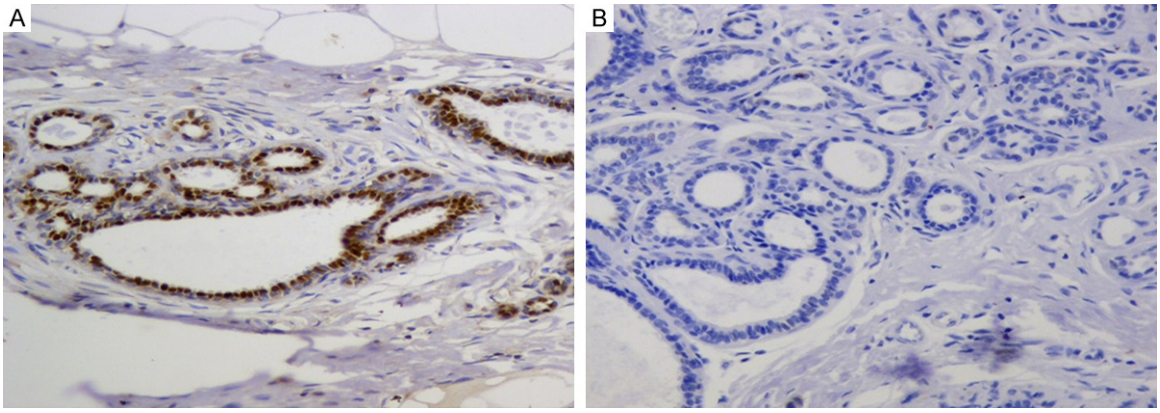

Figure 1. RUNX3 and p21^{waf1} expressions in normal breast tissue. A: RUNX3 showing nuclear staining in the epithelial cells of normal breast (IHC ×200). B: p21^{waf1} showing no detectable staining in the epithelial cells of normal breast (IHC ×200).

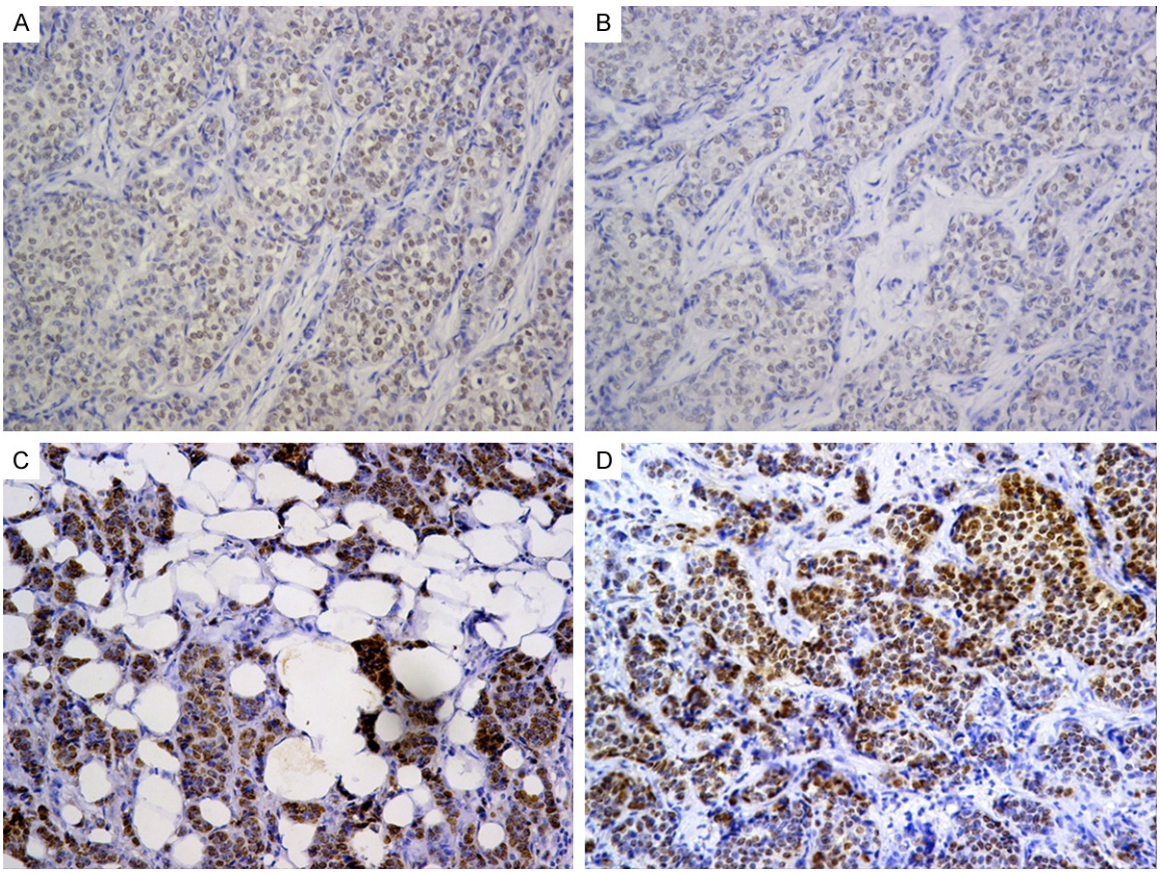

Figure 2. Positive RUNX3 and p21^{waf1} expressions in breast carcinoma. A: RUNX3 showing *low* degree of nuclear expression in a case of invasive duct carcinoma (IHC ×200). B: p21^{waf1} showing *low* nuclear expression in the same case (IHC ×200). C: RUNX3 showing *high* nuclear expression in another case of invasive duct carcinoma (IHC ×200). D: p21^{waf1} showing *high* nuclear expression in the same case as C (IHC ×200).

Results

Clinical and pathological data for the studied breast carcinoma cases are represented in **Table 1**. All patients are females, and their mean age is 53.01 years (Standard deviation, ±5.17; range, 40-64 years).

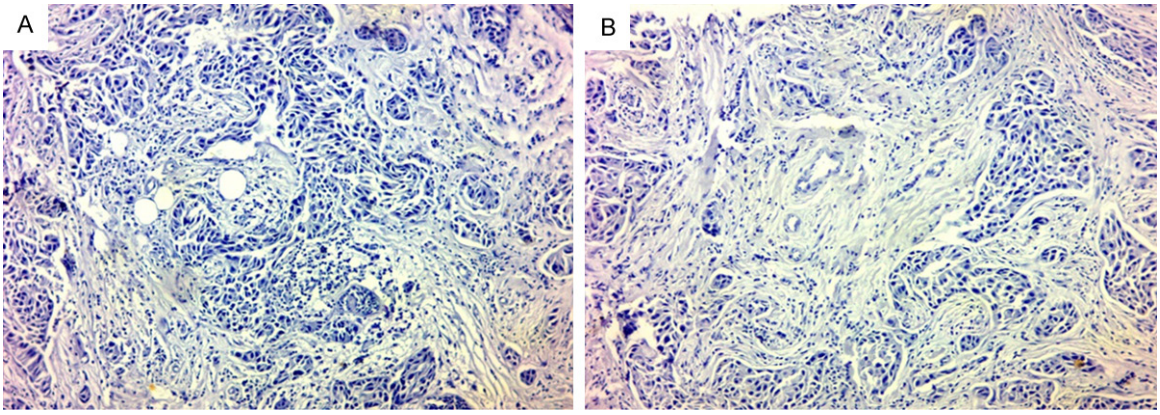

Figure 3. Negative RUNX3 and p21^{waf1} expressions in breast carcinoma. A: RUNX3 showing negative expression in a case of invasive duct carcinoma (IHC×100). B: p21^{waf1} showing negative expression in the same case (IHC×100).

Table 2. Relationship between RUNX3 expression and clinicopathological parameters of the studied breast carcinomas (n=78)

		RUNX3 expression						*P* Value	Sig
		Negative		Low		High			
		N	%	N	%	N	%		
Age	≤50 years	4	17.4%	14	60.9%	5	21.7%	0.483*	NS
	>50 years	10	18.2%	26	47.3%	19	34.5%		
Grade	1	3	21.4%	0	.0%	11	78.6%	0.001**	HS
	2	8	25%	14	43.8%	10	31.2%		
	3	3	9.4%	26	81.2%	3	9.4%		
Lymph nodal status	Negative	8	19.1%	14	33.3%	20	47.6%	0.001*	HS
	Positive	6	16.7%	26	72.2%	4	11.1%		
Size	≤20 mm	6	13.3%	19	42.2%	20	44.4%	0.02**	S
	21-50 mm	7	23.3%	19	63.3%	4	13.3%		
	>50 mm	1	33.3%	2	5.0%	0	.0%		
ER	Negative	7	19.4%	22	61.2%	7	19.4%	0.127*	NS
	Positive	7	16.7%	18	42%	17	40.5%		
PR	Negative	7	19.4%	22	61.2%	7	19.4%	0.127*	NS
	Positive	7	16,7%	18	42%	17	40.5%		
HER2	Negative	9	15%	35	58.3%	16	26.7%	0.074*	NS
	Positive	5	27,8%	5	27.8%	8	44.4%		

*Chi-Square Test, **Fisher exact test, NS = Non Significant, HS = Highly Significant, S = Significant.

Expression of RUNX3 and its relationship with clinicopathological parameter

In normal breast tissue adjacent to the tumor, RUNX3 expression nuclear staining was detected in the epithelial cells of normal acini (**Figure 1A**). On the other hand, 64 (82.1%) out of the 78 invasive ductal breast carcinomas exhibited nuclear RUNX3 expression. Among the positive cases, 40 cases (62.5%) showed low expression (**Figure 2A**), while 24 cases (37.5%) showed high RUNX3 expression (**Figure 2C**). The remaining 14 cases (17.9%) showed negative nuclear expression (**Figure 3A**).

There was a highly statistical significance between RUNX3 expression and both grade and lymph nodal status (P=0.001 for each). Moreover, there was a statistically significant relation with tumor size (P=0.02). However, no statistically significant relationship was found between RUNX3 expression and any of the hor-

Table 3. Relationship between p21^{waf1} expression and clinicopathological parameters of the studied breast carcinomas (n=78)

		p21^{waf1} expression						*P* Value	Sig
		Negative		Low		High			
		N	%	N	%	N	%		
Age	≤50 years	7	30.4%	10	43.5%	6	26.1%	0.787*	NS
	> 50 years	13	23.6%	28	50.9%	14	25.5%		
Grade	1	9	64.3%	1	7.1%	4	28.6%	0.001**	HS
	2	10	31.2%	11	34.4%	11	34.4%		
	3	1	3.1%	26	81.3%	5	15.6%		
Lymph nodal status	Negative	10	23.8%	14	33.3%	18	42.9%	0.001*	HS
	Positive	10	27.8%	24	66.6%	2	5.6%		
Size	≤20 mm	13	28.8%	16	35.6%	16	35.6%	0.045**	S
	21-50 mm	6	20%	20	66.7%	4	13.3%		
	>50 mm	1	33.3%	2	66.7%	0	.0%		
ER	Negative	2	5.6%	24	66.7%	10	27.7%	0.001*	HS
	Positive	18	42.9%	14	33.3%	10	23.8%		
PR	Negative	2	5.6%	24	66.7%	10	27.7%	0.001*	HS
	Positive	18	42.9%	14	33.3%	10	23.8%		
HER2	Negative	18	30%	30	50%	12	20%	0.08**	NS
	Positive	2	11.2%	8	44.4%	8	44.4%		

*Chi-Square Test, **Fisher exact test, NS = Non Significant, HS = Highly Significant, S = Significant.

Table 4. Relationship between RUNX3 expression and breast cancer molecular subtypes (n=78)

		RUNX3 expression						*P* Value	Sig
		Negative		Low		High			
		N	%	N	%	N	%		
Molecular type	Triple negative	4	17.4%	17	73.9%	2	8.7%	0.04**	S
	Her2+ only	3	23%	5	38.5%	5	38.5%		
	Total Luminal*	7	16.6%	18	42.9%	17	40.5%		

*Total Luminal = Luminal' (LUM) subtype + Luminal/HER2+ (LUM/HER2+) subtype, **Fisher exact test, S = Significant.

monal receptors (ER, PR, and Her2 expression). The relationship between clinicopathological parameters and RUNX3 expression is shown in **Table 2**.

Expression of p21^{waf1} and its relationship with clinicopathological parameters

In normal breast tissue adjacent to the tumor, p21^{waf1} expression showed no detectable expression in non-diseased and hyperplastic ductal epithelia (**Figure 1B**), while 58 (74.4%) out of the 78 invasive ductal carcinomas showed positive nuclear p21^{waf1} expression. Among the positive cases, 38 (65.5%) showed low p21^{waf1} expression (**Figure 2B**) and 20 (34.5%) showed high expression (**Figure 2D**). The remaining 20 cases (25.6%) show negative p21^{waf1} expression (**Figure 3B**).

There was a highly statistically significant relationship between p21^{waf1} expression and tumor grade (P=0.001), and lymph nodal status (P= 0.001), and ER expression (P=0.001) as well as PR expression (P=0.001). In addition, there was also a significant relationship between p21^{waf1} expression and tumor size (P=0.045), but there was no statistical correlation between p21^{waf1} expression and HER2 expression. The relationship between clinicopathological parameters and p21^{waf1} expression is shown in **Table 3**.

Relationship between RUNX3 and p21^{waf1} expression and breast cancer molecular subtypes

There was a statistically significant relationship between RUNX3 expression and breast cancer molecular subtypes (P=0.04) (**Table 4**).

Table 5. Relationship between p21waf1 expression and breast cancer molecular subtypes (n=78)

		p21waf1 expression						*P* Value	Sig
		Negative		Low		High			
		N	%	N	%	N	%		
Molecular type	Triple negative	2	8.7%	17	73.9%	4	17.4%	0.001	HS
	Her2+ only	0	.0%	7	53.8%	6	46.2%		
	Total Luminal*	18	42.9%	14	33.3%	10	23.8%		

*Total Luminal = Luminal' (LUM) subtype + Luminal/HER2+ (LUM/HER2+) subtype, HS = Highly Significant.

Table 6. Agreement between immunohistochemical expression of RUNX3 and p21waf1 in breast carcinoma cases (n=78)

		RUNX3 expression						Kappa	Sig
		Negative		Low		High			
		N	%	N	%				
p21waf1 expression	Negative	9	64.3%	4	10.0%	7	29.2%	0.467	0.001 (HS)
	Low	0	.0%	32	80.0%	6	25.0%		
	High	5	35.7%	4	10.0%	11	45.8%		

HS = Highly Significant.

There was a highly significant statistical relation between p21waf1 expression and breast cancer cancer molecular subtypes (P=0.001) (**Table 5**).

Agreement between RUNX3 and p21waf1 expression in invasive ductal breast carcinoma

Comparing RUNX3 and p21waf1 expression in each case, 32 cases showed concomitant low degree of positivity for both RUNX3 and p21waf1 (**Figure 2A**, **2B**), and 11 cases showed concomitant high degree positivity for both markers (**Figure 2C**, **2D**), while 9 cases showed concomitant negative expression for both markers (**Figure 3**). There was a highly statistically significant relation between RUNX3 and p21waf1 expressions (P=0.001), and a moderate significant agreement (kappa =0.467) (**Table 6**).

Discussion

Breast cancer is the most frequently diagnosed cancer and the leading cause of death in females worldwide. It accounted for 23% of the total new cases and 14% of the total cancer deaths in 2008 [2]. Invasive duct carcinoma, the most common type of breast cancer, is a heterogeneous group of tumors that fail to show sufficient characteristics for classification as a specific histological subtype [21].

Despite improvement in early diagnostic methods and advances in treatment, mortality because of breast cancer remains high; there are insufficient information and data about the factors that influence the disease progression and mortality. New prognostic factors such as RUNX3 are becoming valuable tools for the prediction of prognosis and as a potential therapeutic target in women with breast cancer [17].

Several studies have suggested that RUNX3 is a tumor suppressor when its inactivation was seen in numerous types of cancers, such as breast cancer, colorectal cancer, glioma, and melanoma [9]. Like in other cancers, RUNX3 is inactivated in breast cancer by reduced copy number, promoter hypermethylation, hemizygous deletion, and protein mislocalization [8, 21-24]. Consistent with its tumor suppressor activity, reintroduction of RUNX3 into breast cancer cells suppresses their tumorigenic potentials [25-28].

In the current study, the normal breast tissue adjacent to invasive ductal carcinoma showed positive RUNX3 nuclear staining in the epithelial cells lining normal acini. This was in concordance with previous studies [8, 22, 24, 27]. Moreover, 82.1% of invasive ductal breast carcinoma in the present study showed positive RUNX3 expression; among which 62.5% showed low expression, while only 37.5% showed high expression. Previous immunohistochemical studies on RUNX3 expression in breast cancer were conflicting; while Jiang et al.

[24] demonstrated nuclear positivity in 35.2% of their invasive breast carcinoma cases; Subramaniam et al. [8] had only 9% of their cases expressing positive nuclear staining. On the other hand, 68% of Bai et al. [17] cases expressed RUNX3 nuclear positivity.

Only few studies were conducted to assess immunohistochemical expression of RUNX3 in breast carcinoma and heterogeneity in the results within these studies might be attributed to several factors including difference in the commercial company supplying the primary antibody, as well as difference in clonality of the antibody. Moreover, there was a difference in the immunohistochemical scoring in different studies with different cutoff values, where some considered the low expression as negative [8, 22]. Also, it should be noted that each study included different histological types of breast carcinoma. In addition to invasive ductal breast carcinoma, Jiang et al. [24] included invasive lobular carcinoma, and Bai et al. [17], included invasive lobular and other subtypes as well.

In the present work, a high statistically significant correlation was found between RUNX3 expression and lymph nodal status (P=0.001), also a statistically significant relation was present with tumor size (P=0.02); such that high RUNX3 expression occurred in cases with negative lymph nodal affection and small tumor size ≤20 mm. This was in agreement with Jiang et al. [24] who found a significant inverse correlation between RUNX3 expression and lymph nodal status and stage.

Moreover, the current research showed an inverse high statistically significant relationship between RUNX3 expression and the tumor grade; where most of the cases of grade III tumors showed only low RUNX3 expression, whereas high RUNX3 expression occurred more in grade I and II tumors. This was in going with Bai et al. [17] who found out that RUNX3 staining was dramatically decreased in histology grade III compared with histology grade I and II. This was in favor with the tumor suppressing function of RUNX3 in breast cancer as described by Chen [9].

In the current work, there was no statistically significant relationship between RUNX3 expression and either age or hormonal receptors (ER, PR, and Her2 receptor) expression. This is in going with Bai et al. [17].

This study showed a statistically significant relationship between RUNX3 expression and the molecular subtypes of breast carcinoma cases in this research (P=0.04). However, among the triple negative cases in the present work, there was 82.6% RUNX3 positivity. This is in concordance with previous studies that confirmed the increased RUNX3 expression in triple negative tumors [25, 29]. Lau et al. [27] stated that RUNX3 can suppress the tumorigenic potential of estrogen receptor α (ERα) negative breast cancer cells without affecting the proliferation of cells. While the exact mechanism remains unidentified, the anti-tumor activity of RUNX3 in ERα-negative cells might result from RUNX3-mediated cell apoptosis [30, 31] or RUNX3 might target other cellular signaling pathways to exert this anti-tumor activity in these ERα negative cells [9].

Several studies support the tumor suppressor function of RUNX3, through various mechanisms. Hypermethylation of the *Runx3* promoter and cytoplasmic relocalization account for the majority of RUNX3 inactivation in breast cancer [22-24, 27]. It is important to note that these events are reversible and that RUNX3 could be re-activated to restore its tumor suppressing effect [32, 33]. In fact, several studies stated that reintroduction of RUNX3 into RUNX3 deficient breast cancer cells suppressed cancer cell proliferation and their tumorigenic potential [26, 27]. Chen concluded that restoring RUNX3 activation by specific small molecules or inhibitors to block these two events might constitute a novel therapeutic strategy for the treatment of breast cancer [9].

This promising potential therapeutic role of RUNX3 necessitates a better understanding of the exact role of RUNX3 in breast cancer carcinogenesis which remains largely elusive, together with the fact that RUNX3 integrates with various signaling pathways, which play important roles in breast cancer carcinogenesis, as well as the RUNX3 ability to regulate breast cancer cell proliferation, and cell apoptosis as confirmed by several studies [9, 11, 29].

The regulation of RUNX3 shows that the interplay of RUNX3 with p300 and cyclin D1 is the basis of an early defense against tumor forma-

tion. p300 acetylates RUNX3 in response to growth factors, leading to the interaction of RUNX3 with bromodomain-containing protein 2 (BRD2; a member of the BET family of transcription co-regulators) and subsequent transient induction of cyclin dependent kinase inhibitor 1A *(CDKN1A)* and ARF transcription. *CDKN1A* encodes the cell cycle inhibitor p21^{waf1} (also known as CIP1), whereas ARF inhibits MDM2 and thus increases the stability of the tumor suppressor p53. During the cell cycle progression, deacetylation of RUNX3 results in the replacement of the RUNX3-BRD2 complex with a RUNX3-cyclin D1complex and the cessation of *ARF* transcription. However, persistent mitogenic signals from oncogenic KRAS-G12D result in the continued presence of the RUNX3-BRD2 complex and prolonged expression of p21^{waf1}, ARF and p53. RUNX3 might therefore function as a sensor of inappropriate proliferation signals, and its inactivation might induce uncontrolled cell cycle progression and ultimately, cancer [11, 34, 35].

One of the main problems with immunohistochemical studies is that we never know whether RUNX3 overexpression really reflects a genetic abnormality and/or loss of function. A way to investigate the functional status of RUNX3 is therefore to investigate some of its downstream effectors such as p21^{waf1}. Another important issue is the fact that several anticancer agents function through their ability to promote induction of p21^{waf1}. But, the complex network regulating p21^{waf1} activity and biological functions, warrants caution with regard its application for cancer therapy. The various effects of p21^{waf1} on gene regulation and its role in genomic stability, apoptosis, senescence and DNA repair may not only contribute to cancer development but also profoundly affect the efficacy of DNA damaging agents or other anticancer drugs that induce p21^{waf1}. The challenge lies in selectively inhibiting only the oncogenic activities of p21^{waf1} and not its tumor suppressor functions. Therefore, the development of agents that interfere with the ability of p21^{waf1} to assemble CDK4-cyclin D and CDK6-cyclin D complexes but retain its ability to suppress CDK2 or CDK1 may be an attractive line of investigation [36-38].

The current work showed a highly statistically significant relationship as well as a moderately significant agreement between RUNX3 and p21^{waf1} expressions. These results go well with the hypothesis that p21^{waf1} might not be a tumor suppressor itself, but instead it synergizes with tumor suppressors [36, 39-41].

In the present research, the normal breast tissue adjacent to invasive ductal carcinoma showed no detectable p21^{waf1} expression in non-diseased and hyperplastic ductal epithelia as stated by Gohring et al. [42]. Moreover, 74.4% of invasive ductal breast carcinoma in the present study showed positive p21^{waf1} expression; among which 62.5% showed low expression, while only 34.5% showed high expression. This was somewhat near to what Pellikainen et al. [16] demonstrated, where positive p21^{waf1} expression was seen in 71% of their breast cancer cases; among which 38% showed high expression and 62% showed low expression, and also Jiang et al. [43], whose breast carcinoma cases were 39.1% high expression of p21^{waf1} and 68.1% low p21^{waf1} expression. Previous immunohistochemical studies on p21^{waf1} expression in breast cancer were conflicting. While Caffo et al. [44] and Barbareschi et al. [45] demonstrated 82% and 90% p21^{waf1} positivity, respectively, Gohring et al. [42] only demonstrates 32.2% positivity in his invasive breast carcinoma cases.

In this study, there was a highly statistically significant relationship between tumor grade and p21^{waf1} expression (P=0.001); such that 53.4% of grade 3 cases showed positive p21^{waf1} expression, of which 68.4% showed only low p21^{waf1} expression. In this context, previous studies showed contradictory results; some authors associated high p21^{waf1} expression with poor differentiation [16, 44, 46], whereas others have found it to be associated with increased differentiation [47, 48]. This may be due to different patient samples, antibodies, analyzing methods and cut off values used in these p21^{waf1} studies, which make comparisons of findings difficult. Moreover, as stated by Abbas and Dutta [36], the simple view that p21^{waf1} acted as a tumor suppressor had been complicated by the finding that p21^{waf1} could exhibit oncogenic activities.

The current study finds a highly statistically significant relation between p21^{waf1} and lymph nodal status (P=0.001). In addition, there was

a statistically significant relation with tumor size (P=0.045); where higher p21^{waf1} expression correlated with smaller sizes. This was in concordance with Caffo et al. [44].

The present work shows a highly statistically significant relationship between p21^{waf1} expression and estrogen receptor as well as progesterone receptor expression (P=0.001 for each), this is in agreement with Pellikainen et al. [16], who found a statistically significant relationship between p21^{waf1} expression and hormone receptor status in breast cancer. There was also a high statistically significant relationship between the molecular subtypes of the breast carcinoma cases in the current study and p21^{waf1} expression (P=0.001). Among the TNP cases, there was 91.3% positive p21^{waf1} nuclear expression.

However, in this present work, there was no statistically significant relationship between p21^{waf1} and Her2 expression, unlike Winters et al. [49] who found a weak inverse association between nuclear p21^{waf1} and Her2 expression, Moreover, Winters et al. breast carcinoma cases demonstrated cytoplasmic expression of p21^{waf1} which showed a stronger direct statistical relationship with Her2. They suggested that p21^{waf1} could localize in the cytoplasm in cancer tissues and cell lines, where it inhibited apoptosis by binding and inhibiting the apoptosis signal-regulating kinase 1, and that such an anti-apoptotic function in breast cancers could underlie the association between cytoplasmic p21^{waf1} and poor prognosis. On the other hand, breast carcinoma cases in the current study, like many previous studies [42, 44, 45], showed no cytoplasmic expression of p21^{waf1}.

In conclusion, the current study reports for the first time the relationship between RUNX3 and p21^{waf1} immunohistochemical expression in breast cancer. The two markers are statistically related to established prognostic clinicopathologic parameters suggesting a role in prognosis. They also represent potential therapeutic targets for patients with breast cancer.

A limitation of this study is that cases were derived from a regional population base that lacks breast cancer outcomes including response to different modalities of therapy and disease-free survival. Therefore, further larger studies are still needed to explore the direct relation of RUNX3 and p21^{waf1} expression to breast cancer patients' survival and their prognostic value within different treatment modality subsets.

Disclosure of conflict of interest

None.

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References

[1] Parkin DM and Fernandez LM. Use of statistics to assess the global burden of breast cancer. Breast J 2006; 12: S70-S80.

[2] Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61: 69-90.

[3] In: Mokhtar N, Gouda I and Adel I, editors. Cancer pathology registry 2003-2004 and time trend analysis. Cairo University, Egypt: National Cancer Institute; 2007.

[4] Schnitt SJ. Classsification and prognosis of invasive breast cancer: from morphology to molecular taxonomy. Mod Pathol 2010; 23: S60-S64.

[5] Chuang LS, Ito K, Ito Y. RUNX family: Regulation and diversification of roles through 6 interacting proteins. Int J Cancer 2013; 132: 1260-1271.

[6] Blyth K, Cameron ER, Neil JC. The RUNX genes: gain or loss of function in cancer. Nat Rev Cancer 2005; 5: 376-387.

[7] Li QL, Ito K, Sakakura C, Fukamachi H, Inoue K, Chi XZ, Lee KY, Nomura S, Lee CW, Han SB, Kim HM, Kim WJ, Yamamoto H, Yamashita N, Yano T, Ikeda T, Itohara S, Inazawa J, Abe T, Hagiwara A, Yamagishi H, Ooe A, Kaneda A, Sugimura T, Ushijma T, Bae SC, Ito Y. Causal relationship between the loss of RUNX3 expression and gastric cancer. Cell 2002; 109: 113-124.

[8] Subramaniam MM, Chan JY, Yeoh KG, Quek T, Ito K, Salto-Tellez M. Molecular pathology of RUNX3 in human carcinogenesis. Biochem Biophys Acta 2009; 1796: 315-31.

[9] Chen LF. Tumor suppressor function of RUNX3 in breast cancer. J Cell Biochem 2012; 113: 1470-1477.

[10] Bos R, van Diest PJ, van der Groep P, Shvarts A, Greijer AE, van der Wall E. Expression of hypoxia-inducible factor-1 alpha and cell cycle proteins in invasive breast cancer are estrogen

receptor related. Breast Cancer Res 2004; 6: R450-9.
[11] Ito Y, Bae SC, Chuang LS. The RUNX family: developmental regulators in cancer. Nat Rev Cancer 2015; 15: 81-95.
[12] Singletary SE, Allred C, Ashley P, Bassett LW, Berry D, Bland KI, Borgen PI, Clark GM, Edge SB, Hayes DF, Hughes LL, Hutter RV, Morrow M, Page DL, Recht A, Theriault RL, Thor A, Weaver DL, Wieand HS, Greene FL. Staging system for breast cancer: revisions for the 6th edition of the AJCC Cancer Staging Manual. Surg Clin North A 2003; 83: 803-819.
[13] Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991; 19: 403-410.
[14] Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. J Histochem Cytochem 1981; 29: 577-580.
[15] Simon R, Altman DG. Statistical aspects of prognostic factor studies in oncology. Br J Cancer 1994; 69: 979-85.
[16] Pellikainen MJ, Pekola TT, Ropponen KM, Kataja VV, Kellokoski JK, Eskelinen MJ, Kosma VM. p21WAF1 expression in invasive breast cancer and its association with p53, AP-2, cell proliferation, and prognosis. J Clin Pathol 2003; 56: 214-220.
[17] Bai J, Yong HM, Chen FF, Song WB, Li C, Liu H, Zheng JN. RUNX3 is a prognostic marker and potential therapeutic target in human breast cancer. Cancer Res Clin Oncol 2013; 139: 1813-1823.
[18] Araki K, Osaki M, Nagahama Y, Hiramatsu T, Nakamura H, Ohgi S, Ito H. Expression of RUNX3 protein in human lung adenocarcinoma: Implications for tumor progression and prognosis. Cancer Sci 2005; 96: 227-231.
[19] Cheang MC, Voduc D, Bajdik C, Leung S, McKinney S, Chia SK, Perou CM, Nielsen TO. Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. Clin Cancer Res 2008; 14: 1368-1376.
[20] In: Tavassoeli FA and Devilee P, editors. World Health Organization: Tumours of the breast and female genital organs. 1st edition. Lyon, France: IARC Press; 2003.
[21] Chen W, Salto-Tellez M, Palanisamy N, Ganesan K, Hou Q, Tan LK, Sii LH, Ito K, Tan B, Wu J, Tay A, Tan KC, Ang E, Tan BK, Tan PH, Ito Y, Tan P. Targets of genome copy number reduction in primary breast cancers identified by integrative genomics. Genes Chromosomes Cancer 2007; 46: 288-301.
[22] Subramaniam MM, Chan JY, Soong R, Ito K, Ito Y, Yeoh KG, Salto-Tellez M, Putti TC. RUNX3 inactivation by frequent promoter hypermethylation and protein mislocalization constitute an early event in breast cancer progression. Breast Cancer Res Treat 2009; 113: 113-121.
[23] Hwang KT, Han W, Bae JY, Hwang SE, Shin HJ, Lee JE, Kim SW, Min HJ, Noh DY. Downregulation of the RUNX3 gene by promoter hypermethylation and hemizygous deletion in breast cancer. J Korean Med Sci 2007; 22: S24-S31.
[24] Jiang Y, Tong D, Lou G, Zhang Y, Geng J. Expression of RUNX3 gene, methylation status and clinicopathological significance in breast cancer and breast cancer cell lines. Pathobiology 2008; 75: 244-251.
[25] Huang B, Qu Z, Ong CW, Tsang Y-HN, Xiao G, Shapiro D, Salto-Tellez M, Ito K, Ito Y, Chen LF. RUNX3 acts as a tumor suppressor in breast cancer by targeting estrogen receptor α. Oncogene 2012; 31: 527-534.
[26] Huang N, Parantu K, Shah PK, Li C. Lessons from a decade of integrating cancer copy number alterations with gene expression profiles. Brief Bioinform 2011; 13: 305-16.
[27] Lau QC, Raja E, Salto-Tellez M, Liu Q, Ito K, Inoue M, Putti TC, Loh M, Ko TK, Huang C, Bhalla KN, Zhu T, Ito Y, Sukumar S. RUNX3 is frequently inactivated by dual mechanisms of protein mislocalization and promoter hypermethylation in breast cancer. Cancer Res 2006; 66: 6512-652.
[28] Wilting RH, Dannenberg JH. Epigenetic mechanisms in tumorigenesis, tumor cell heterogeneity and drug resistance. Drug Resistance Updates 2012; 15: 21-38.
[29] Chimge NO, Frenkel B. The RUNX family in breast cancer: relationships with estrogen signaling. Oncogene 2013; 32: 2121-2130.
[30] Yamamura Y, Lee WL, Inoue K, Ida H, Ito Y. RUNX3 cooperates with Foxo3a to induce apoptosis in gastric cancer cells. J Biol Chem 2006; 281: 5267-76.
[31] Yano T, Ito K, Fukamachi H, Chi XZ, Wee HJ, Inoue K, Ida H, Bouillet P, Strasser A, Bae SC, Ito Y. The RUNX3 tumor suppressor upregulates Bim in gastric epithelial cells undergoing transforming growth factor beta-induced apoptosis. Mol Cell Biol 2006; 26: 4474-88.
[32] Goh YM, Cinghu S, Hong ET, Lee YS, Kim JH, Jang JW, Li YH, Chi XZ, Lee KS, Wee H, Ito Y, Oh BC, Bae SC. Src kinase phosphorylates RUNX3 at tyrosine residues and localizes the protein in the cytoplasm. J Biol Chem 2010; 285: 10122-9.
[33] Guo WH, Weng LQ, Ito K, Chen LF, Nakanishi H, Tatematsu M, Ito Y. Inhibition of growth of

mouse gastric cancer cells by Runx3, a novel tumor suppressor. Oncogene 2002; 21: 8351-5.
[34] Belkina AC, Denis GV. BET domain co-regulators in obesity, inflammation and cancer. Nature Rev Cancer 2012; 12: 465-477.
[35] Lee YS, Lee JW, Jang JW, Chi XZ, Kim JH, Li YH, Kim MK, Kim DM, Choi BS, Kim EG, Chung JH, Lee OJ, Lee YM, Suh JW, Chuang LS, Ito Y, Bae SC. Runx3 inactivation is a crucial early event in the development of lung adenocarcinoma. Cancer Cell 2013; 24: 603-616.
[36] Abbas T, Dutta A. p21 in cancer: intricate networks and multiple activities. Nat Rev Cancer 2009; 9: 400-414.
[37] Ocker M, Schneider-Stock R. Histone deacetylase inhibitors: signalling towards p21cip1/waf1. Int J Biochem Cell Biol 2007; 39: 1367-1374.
[38] Ukomadu C, Dutta A. p21-dependent inhibition of colon cancer cell growth by mevastatin is independent of inhibition of G1 cyclin-dependent kinases. J Biol Chem 2003; 278: 43586-43594.
[39] Shiohara M, El-Deiry WS, Wada M, Nakamaki T, Takeuchi S, Yang R, Chen DL, Vogelstein B, Koeffler HP. Absence of WAF1 mutations in a variety of human malignancies. Blood 1994; 84: 3781-3784.
[40] McKenzie KE, Siva A, Maier S, Runnebaum IB, Seshadri R, Sukumar S. Altered WAF1 genes do not play a role in abnormal cell cycle regulation in breast cancers lacking p53 mutations. Clin Cancer Res 1997; 3: 1669-1673.
[41] Patino-Garcia A, Sotillo-Pineiro E, Sierrasesumaga-Ariznabarreta L. p21waf1 mutation is not a predominant alteration in pediatric bone tumors. Pediatr Res 1998; 43: 393-395.
[42] Göhring UJ, Bersch A, Becker M, Neuhaus W, Schöndorf T. p21waf correlates with DNA replication but not with prognosis in invasive breast cancer. J Clin Pathol 2001; 54: 866-870.
[43] Jiang M, Shao ZM, Wu J, Lu JS, Yu LM, Yuan JD, Han QX, Shen ZZ, Fontana JA. p21/waf1/cip1 and mdm-2 expression in breast carcinoma patients as related to prognosis. Int J Cancer (Pred Oncol) 1997; 74: 529-534.
[44] Caffo O, Doglioni C, Veronese S, Bonzanini M, Marchetti A, Buttitta F, Fina P, Leek R, Morelli L, Dalla Palma P, Harris AL, Barbareschi M. Prognostic value of p21waf1 and p53 expression in breast carcinoma: an immunohistochemical study in 261 patients with long-term follow-up. Clin Cancer Res 1996; 2: 1591-1599.
[45] Barbareschi M, Caffo O, Doglioni C, Fina P, Marchetti A, Buttitta F, Leek R, Morelli L, Leonardil E, Bevilacqua G, Dalla Palma P, Harris AL. p21waf1 immunohistochemical expression in breast carcinoma: correlations with clinicopathological data, oestrogen receptor status, MIB1 expression, p53 gene and protein alterations and relapse-free survival. Br J Cancer 1996; 74: 208-215.
[46] Rey MJ, Fernandez PL, Jares P, Muñoz M, Nadal A, Peiró N, Nayach I, Mallofré C, Muntané J, Campo E, Estapé J, Cardesa A. p21WAF1/Cip1 is associated with cyclin D1CCND1 expression and tubular differentiation but is independent of p53 overexpression in human breast carcinoma. J Pathol 1998; 184: 265-71.
[47] Wakasugi E, Kobayashi T, Tamaki Y, Ito Y, Miyashiro I, Komoike Y, Takeda T, Shin E, Takatsuka Y, Kikkawa N, Monden T, Monden M. p21(Waf1/Cip1) and p53 protein expression in breast cancer. Am J Clin Pathol 1997; 107: 684-691.
[48] Domagala W, Welcker M, Chosia M, Karbowniczek M, Harezga B, Bartkova J, Bartek J, Osborn M. p21/WAF1/Cip1 expression in invasive ductal breast carcinoma: relationship to p53, proliferation rate, and survival at 5 years. Virchows Arch 2001; 439: 132-40.
[49] Winters ZE, Leek RD, Bradburn MJ, Norbury CJ, Harris AL. Cytoplasmic p21WAF1/CIP1 expression is correlated with HER-2/neu in breast cancer and is an independent predictor of prognosis. Breast Cancer Res 2003; 5: R242-R249.